
A Gradient of Molecules in Avian Retina with Dorsoventral Polarity

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• Mechanisms that impart positional information for the assembly of the developing nervous system and the establishment of specific synaptic connections have been studied in model systems. The retina is a highly ordered laminar structure due to segregation of different classes of neuronal cell bodies and synapses into separate strata (Karten, this volume). Synaptic connections between retina ganglion neurons and tectum neurons preserve the topographic relations of ganglion neurons in the retina, resulting in a point-to-point retino-tectal map. However, the mechanisms underlying these phenomena have not been defined. Sperry (1963) postulated that two orthogonal gradients of molecules on retina ganglion neurons and corresponding gradients of complementary molecules in the optic tectum might determine the specificity of connections between retina and tectum neurons. Other mechanisms that have been proposed include adhesive interactions between migrating retina neurites, myelination of bundles of retina axons, and formation of extracellular channels by glia to guide retina axons (Silver and Sidman 1980).

Antisera have been widely used to study retinal structure and function (Goldschneider and Moscona 1972; Thiery et al. 1977; Hausman and Moscona 1979). Clonal neural retina hybrid cell lines derived from single retina cells (as a homogeneous immunogen) have been used to produce rabbit antiserum demonstrating an antigen of the rodent neural retina that was expressed in a restricted topographic domain (Trisler et al. 1979). Hybridoma cell lines were derived by fusing mouse myeloma cells with spleen cells from mice immunized with retina cells to produce monoclonal antibodies to retina neurons or Müller cells (Eisenbarth et al. 1979; Barnstable 1980).

To detect cell-surface molecules with topographic specificity in the retina, hybridoma antibodies, as candidates for neuronal recognition molecules, were obtained by fusing P3-X63/Ag-8 mouse myeloma cells (Köhler and Milstein 1975) with spleen cells from mice immunized with small portions of dorsal or ventral 14-day chick embryo neural retina or clonal retina hybrid cells. A hybridoma antibody was obtained that binds to cell-membrane molecules distributed in a dorsoposterior \rightarrow ventroanterior gradient in retina (Trisler et al. 1981).

DETECTION OF ANTIBODIES WITH TOPOGRAPHIC SPECIFICITY

• The rationale for the experiments shown schematically in Figure 1 was to immunize mice with cells from dorsoposterior or ventral 14-day chick embryo left retina to obtain hybridoma cell lines synthesizing antibody with specificity for the retina sector used for immunization. The choroid fissure was used as a landmark. Female BALB/c

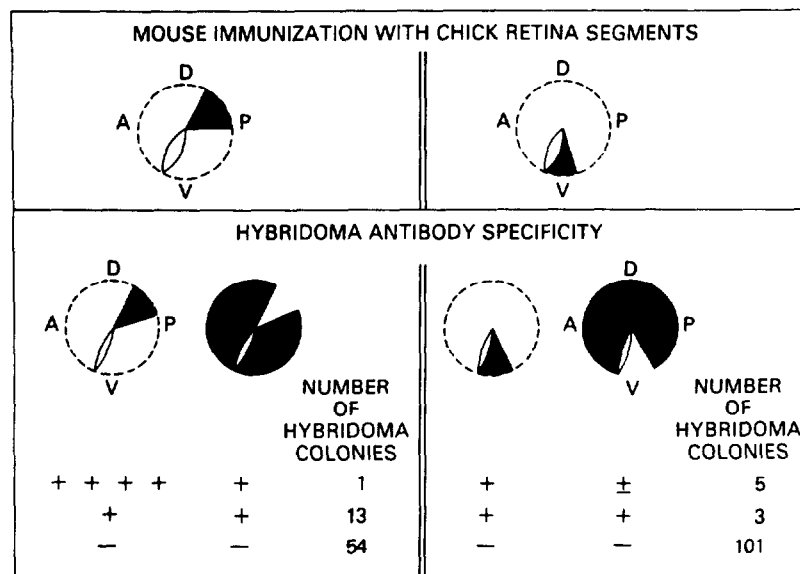


Figure 1
Strategy for detecting region-specific retina surface-membrane antigens. Lymphocyte hybridomas were derived from mice immunized with cells from dorsoposterior (left panels) or ventral (right panels) retina; hybridoma antibody binding to paired cultured-cell monolayers from the portions of 14-day chick embryo retina shown in black in the lower panels was determined.

mice were immunized on days 0, 7, and 14 intraperitoneally with 8×10^6 mechanically dissociated retina cells and intravenously with 2×10^6 cells from dorsoposterior or ventral retina. On the 17th day, 1×10^8 spleen cells from an immunized mouse were fused with 2×10^7 P3-X63/Ag-8 mouse myeloma cells as described by Galfre et al. (1977). After fusion, the cells were suspended with 5×10^6 spleen cells from a nonimmunized mouse in 50 ml of medium (Trisler et al. 1981), and 672 wells of 96-well microculture plates were inoculated with cells ($74 \mu\text{l}/\text{well}$). Additional medium was added ($0.1 \text{ ml}/\text{well}$) on the 5th and 10th days after fusion.

To detect an antibody to a surface molecule enriched in one region of the retina, tissue culture supernatants from wells with hybridoma colonies were tested by indirect radioimmunoassay against cells cultured for 2 days (Schneider and Eisenbarth 1979);

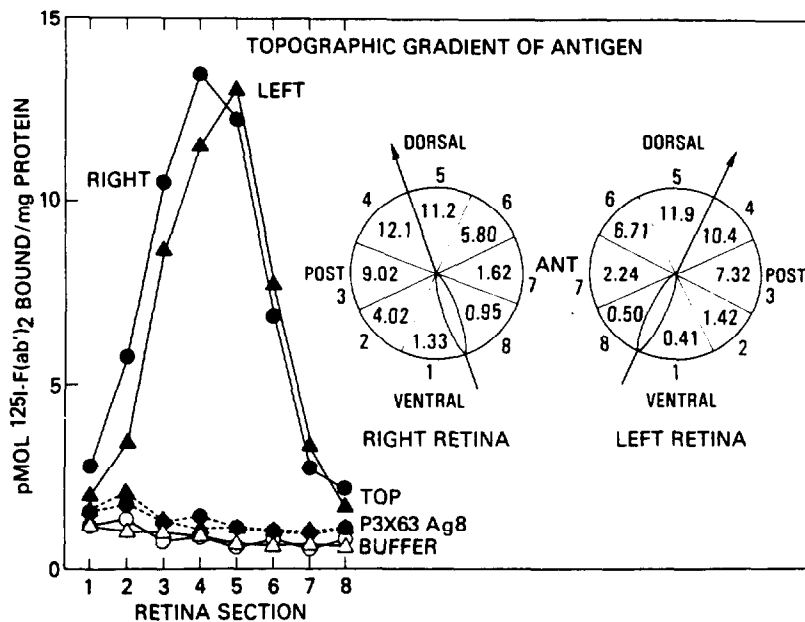


Figure 2

Topographic gradient of TOP antigen in (●) right or (▲) left 14-day chick embryo neural retina. Equivalent sectors from different retinas were pooled and assayed for [^{125}I]F(ab')₂ binding after incubation with (●—●; ▲—▲) TOP antibody, (●-----●; ▲-----▲) P3-X63/Ag-8 antibody, or (○—○, Δ—Δ) buffer in the absence of antibody. The right half of the figure depicts, within each sector of the retina, the pmoles of specifically bound [^{125}I]F(ab')₂ per mg of retina protein (binding due to antibody to TOP minus binding due to antibody synthesized by P3-X63/Ag-8 parental cells). Assay conditions are described in the legend to Fig. 3.

cells were taken from the one eighth of the retina originally used as immunogen or from the remaining seven eighths of the retina. Hybridoma antibody binding to retina cells was detected with an ^{125}I -labeled, affinity-purified $\text{F}(\text{ab}')_2$ fragment of rabbit anti-mouse IgG ($^{125}\text{I}[\text{F}(\text{ab}')_2]$).

A TOPOGRAPHIC GRADIENT OF SURFACE-MEMBRANE MOLECULES

- One of 155 hybridoma cell lines examined synthesized antibody to a cell-surface antigen distributed preferentially in dorsoposterior retina (Fig. 2), designated TOP, for toponymic antigen (i.e., a marker of position). Neural retinas from right or left eyes of 14-day chick

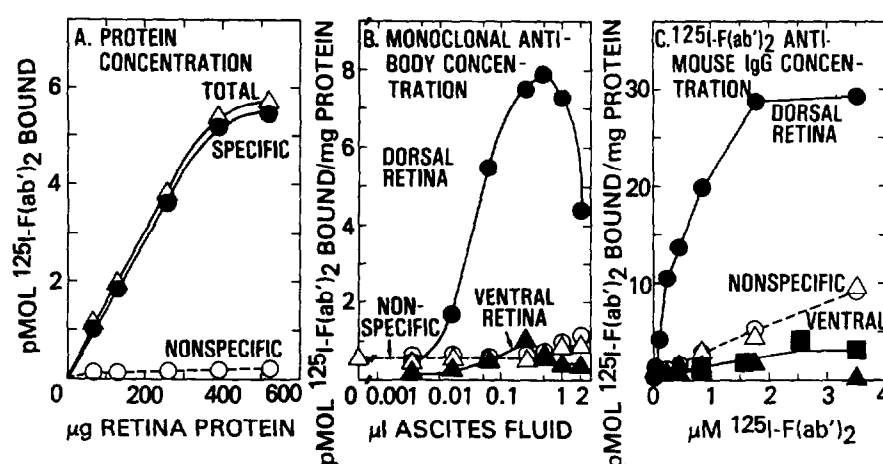


Figure 3

(A) Effect of dorsal retina protein concentration (pooled sections 4 and 5 from 14-day chick embryos as illustrated in Fig. 2) on $^{125}\text{I}[\text{F}(\text{ab}')_2]$ binding due to: (Δ) antibody to TOP; (\circ) antibody synthesized by P3-X63/Ag-8; (\bullet) specific binding. (B) Effect of monoclonal antibody concentration on (\bullet , \blacktriangle) $^{125}\text{I}[\text{F}(\text{ab}')_2]$ specific binding due to antibody to TOP or (\circ , Δ) nonspecific binding due to P3-X63/Ag-8 antibody, to cells from (\bullet) dorsal retina sections 4 and 5 or (\blacktriangle) ventral retina sections 1 and 8. (C) Effect of concentration of $^{125}\text{I}[\text{F}(\text{ab}')_2]$ rabbit anti-mouse IgG. For all experiments each reaction mixture contained the following components, except where specified, in a final volume of 50 μl : 150–200 μg of retina protein, 50 μg of gelatin, and 1 μl of hybridoma ascites fluid; the reaction mixtures were incubated for 30 min at 4°C and washed three times. The pellets were resuspended in 50 μl containing 440 nM $^{125}\text{I}[\text{F}(\text{ab}')_2]$ (50,000 cpm), 50 μg of gelatin, and 500 μg of bovine serum albumin in Dulbecco's phosphate-buffered saline, incubated for 30 min at 4°C, washed three times, and counted.

embryos were cut into eight 45° sectors and assayed for TOP antigen. The highest concentration of antigen detected was in dorsoposterior retina and the lowest in ventroanterior retina. Bilaterally symmetric gradients were found in right and left eyes. Other hybridoma antibodies, including A2B5 (Eisenbarth et al. 1979), bound equally to cells from each region of retina.

The effects of varying concentrations of retina protein, hybridoma antibody, and [125 I]F(ab')₂ rabbit anti-mouse IgG are shown in Figure 3, A, B, and C, respectively. Assay conditions employed are summarized in the legend to Figure 3.

To determine whether the TOP antigen gradient is polar (i.e., varies with angle of rotation around the center of the retina), with

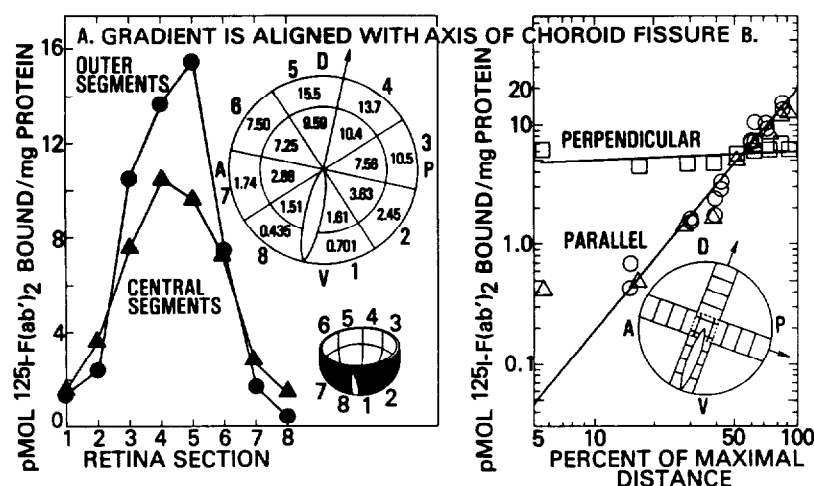


Figure 4

Orientation of the TOP gradient. Specific binding of [125 I]F(ab')₂ (pmoles per mg of protein) is shown within the appropriate segment of retina in A and on the ordinate in A and B. (A) Each left retina was cut into eight 45° sectors (7.25 mm in length), which were divided into central (4.9 mm) and outer (2.35 mm) segments. (B) Demonstration that TOP concentration detected is a function of the square of distance from the ventroanterior margin of the retina. (Δ) Strips of retina 2.5 mm wide running from the ventroanterior margin (0% distance) to the dorsoposterior margin of the retina (14.5 mm = 100% of maximal distance), parallel to the choroid fissure, were removed from eight retinas (left eyes) and each was cut into nine 1.5-mm segments as shown. (\square) Strips of retina from anterior (0%) to posterior (100%) margins of retina were prepared and assayed as above. (\circ) The data from A. The length of the arc from the ventral pole of the gradient to the center of each segment was calculated assuming the retina to be a hemisphere and using equations relating angles and sides of spherical triangles.

uniform antigen concentration along any line of radius, or varies with distance from the dorsoposterior to the ventroanterior margins of the retina, left retinas of 14-day chick embryos were divided into 16 sections as shown in Figure 4A and assayed for TOP. Specific [^{125}I]F(ab')₂ binding varied from 0.35 pmoles per mg of protein at the ventroanterior margin to 15.5 pmoles per mg of protein at the dorsoposterior margin of the retina. Thus, a 35-fold gradient of TOP antigen was detected, aligned with the dorsoposterior-ventroanterior axis of the eye (i.e., oriented parallel to the long axis of the choroid fissure).

Antigen concentration detected was a logarithmic function of distance from the dorsoposterior pole of the gradient to the ventroanterior pole along the circumference of the retina (Fig. 4B). In contrast, little or no variation in TOP antigen concentration was detected along the axis perpendicular to the choroid fissure. The concentration of [^{125}I]F(ab')₂•anti-TOP antibody•TOP antigen complex detected (F_x) is a function of the square of the circumferential distance (D_x) from the ventroanterior pole of the gradient toward the dorsoposterior pole. Thus, TOP molecules can be used as a marker of cell position along a ventroanterior-dorsoposterior axis of retina, i.e.,

$$D_x = D_{\max} (F_x/F_{\max})^{0.5},$$

where D_{\max} and F_{\max} are maximal values for retina at the dorsoposterior margin of the retina. Under the conditions used for the experiments shown in Figure 4, A and B, F_{\max} was 20 pmoles of [^{125}I]F(ab')₂ bound specifically per mg of protein, and D_{\max} was 14.5 mm. Thus, the calculated mean position in retina of cells that bind 5 pmoles of [^{125}I]F(ab')₂ specifically per mg of protein is 7.25 mm from the ventroanterior pole of the gradient, which agrees well with the experimentally determined values.

EXPRESSION OF TOP ANTIGEN DURING DEVELOPMENT

- The antigen was detected in the optic cups of 48-hour chick embryos, and evidence for a gradient of TOP in retina was found with 4-day chick embryos, the earliest stage tested, through the adult (Fig. 5). Whereas the amount of TOP detected per mg of ventral retina protein remained constant throughout development, the concentration of TOP antigen in dorsal retina increased threefold between the 4th and 12th days of embryonic development and then decreased somewhat in the adult. In summary, these results suggest that a gradient of TOP molecules is formed early in retina development, during active neuroblast proliferation and neuron genesis, and is maintained after neuron genesis ceases.

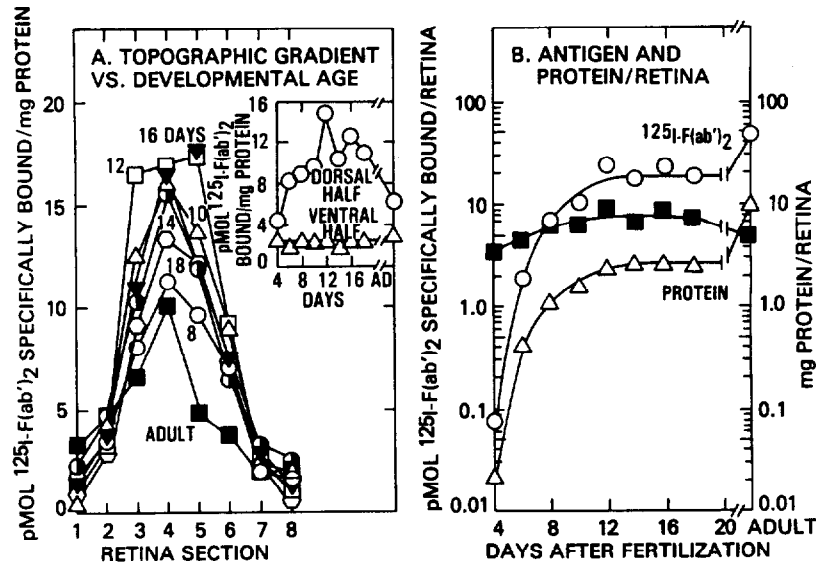


Figure 5

Expression of TOP antigen in chick retina during embryonic development and in the adult. (A) Symbols represent antigen concentration detected in each of eight radial sectors of retina (shown in Fig. 2) from chick embryos at: (○) day 8, (Δ) 10, (□) 12, (●) 14, (▼) 16, (⊙) 18, and (■) adult. The insert depicts antigen detected in dorsal or ventral halves of retina from 4- through 18-day embryos and adults. (B) (○) TOP antigen detected per retina; (Δ) protein per retina; (■) $^{125}\text{I-F(ab')}_2$ specifically bound per mg of protein.

A chick embryo was found with a third eye in the middle of the forehead (Fig. 6); retinas from the right, middle, and left eyes each had a gradient of TOP molecules with normal symmetry, polarity, and orientation. Thus, a gradient of TOP with normal orientation was generated in the third eye despite the abnormal orientation of the eye in the embryo.

ANTIGEN DISTRIBUTION

- The antigen was detected by autoradiography (Fig. 7) or indirect immunofluorescence on most, if not all, cell types in 14-day chick embryo dorsoposterior retina. More antigen was detected in the inner and outer synaptic regions than elsewhere in the retina. Little antigen was detected in ventroanterior retina. All mechanically dissociated cells examined from 8-day chick embryo dorsoposterior retina had punctate rim fluorescence; all cells examined from middle retina also

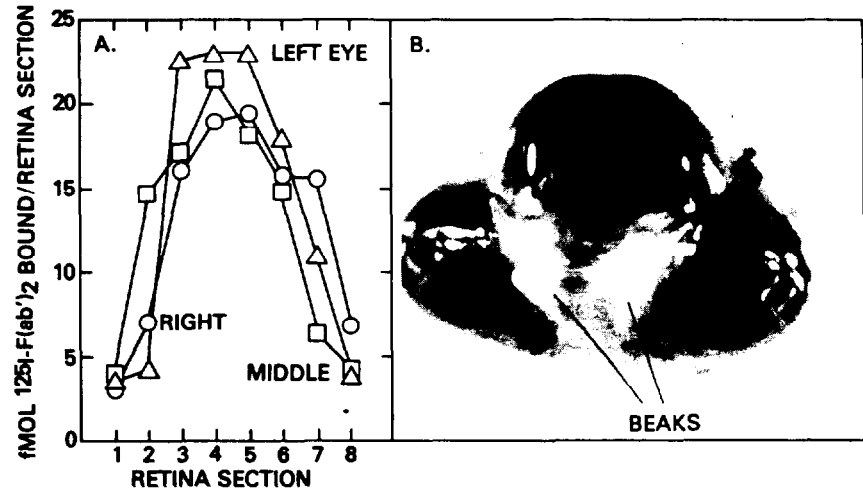


Figure 6

(A) TOP antigen gradients in retinas from the (○) right, (□) middle, and (Δ) left eyes of a 14-day chick embryo with three eyes. Total [^{125}I]F(ab')₂ bound per retina section is shown. Reaction mixtures contained 2.38 nM [^{125}I]F(ab')₂. (B) Frontal view of head of embryo. The third eye is situated on the forehead and oriented in a dorsoanterior direction. The embryo had two pairs of beaks, two brains in one head, and one body.

were fluorescent, but less intensely than dorsoposterior retina cells. No fluorescent cells were detected in ventroanterior retina.

The highest concentrations of TOP antigen were found in tissue derived from the forebrain (Table 1): retina > cerebrum > thalamus. Lower levels of antigen were found in optic nerve, cerebellum, dorsal and ventral retina pigment epithelium, and optic tectum. Little or no antigen was detected in heart, liver, kidney, or blood cells.

Gradients of TOP molecules with similar orientation and symmetry were detected in turkey, quail, and duck retina (Fig. 8), but the antigen was not detected in goldfish, *Xenopus laevis*, *Rana pipiens*, or postnatal Fisher rat retina.

PROPERTIES OF TOP ANTIGEN

- Incubation of 14-day chick embryo retina cells at 100°C or incubation at 37°C with 11 μM trypsin resulted in loss of antigenic activity from cells (Table 2). To study the possible involvement of carbohydrate residues, hapten-competition experiments were performed (Table 2). Incubation of retina cells with wheat germ agglutinin (which binds to β -N-acetylglucosaminyl residues) at 4°C reduced

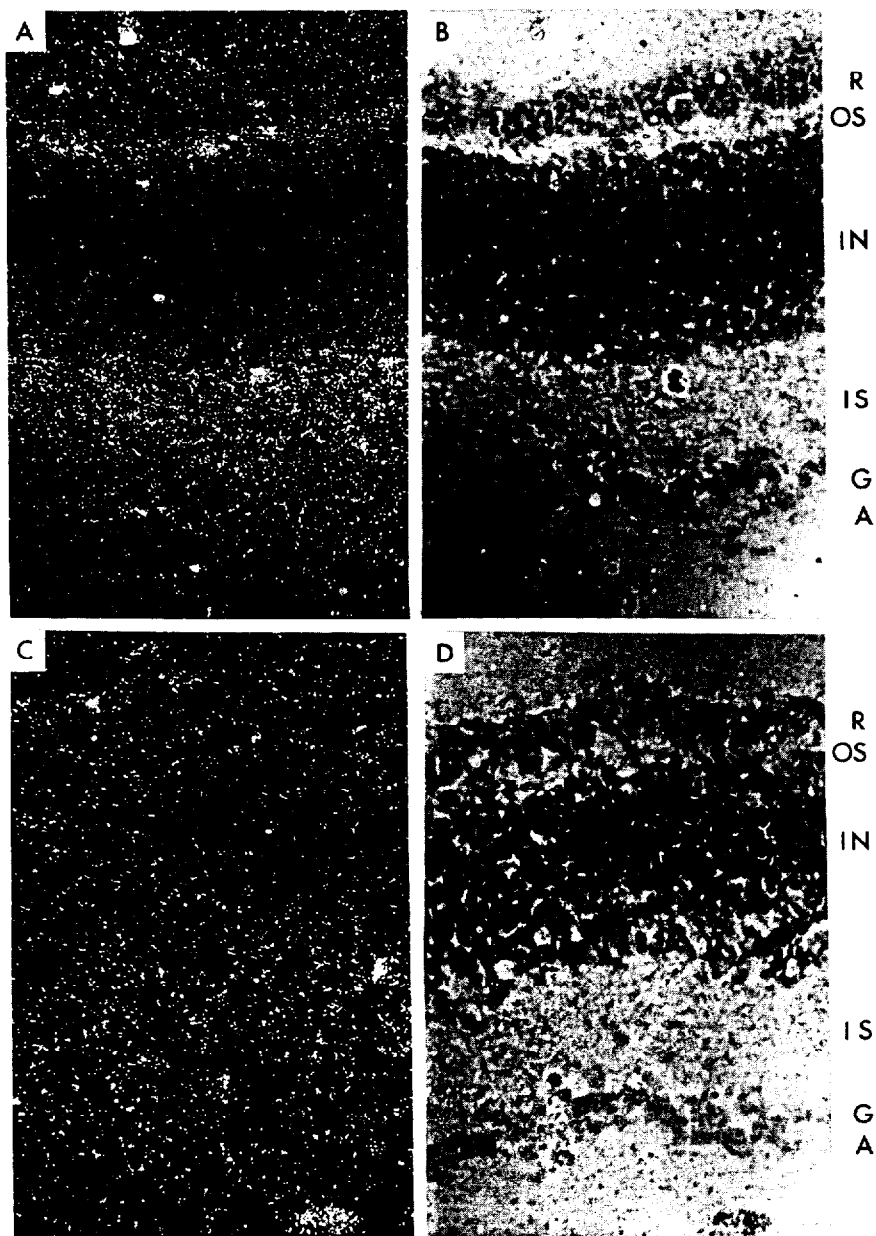


Figure 7

Autoradiographs of 14-day chick embryo retina. Frozen sections ($16\ \mu\text{m}$) from outer halves of dorsal or ventral retina were treated with antibody to TOP, followed by $[^{125}\text{I}]\text{F(ab')}_2$, exposed 22 days, and stained with toluidine blue. (A) Dark-field and (B) phase-contrast views of dorsal retina. In A, some silver grains over cell soma in the inner nuclear layer appear dim due to staining of cells by toluidine blue. (C) Dark-field and (D) phase-contrast views of ventral retina. (R) photoreceptor layer; (OS) outer synaptic layer; (IN) inner nuclear layer; (IS) inner synaptic layer; (G) ganglion cell layer; (A) ganglion cell axon layer. Magnification, $517\times$.

Table 1
Distribution of TOP Antigen in Chicken Tissues

| Tissue | 14-Day embryo (pmoles [125 I]F(ab') $_2$ specifically bound/mg protein) | Adult |
|--------------------------------------|----------------------------------------------------------------------------------|-------|
| Dorsal neural retina | 12.0 | 7.70 |
| Ventral neural retina | 1.08 | 2.70 |
| Cerebrum | 3.30 | 3.12 |
| Thalamus | 2.13 | — |
| Optic nerve | — | 0.58 |
| Optic tectum | 0.15 | — |
| Cerebellum | 0.23 | 0.46 |
| Dorsal retina pigment epithelium | 0.26 | — |
| Ventral retina pigment epithelium | 0.25 | — |
| Heart, liver, kidney, or blood cells | 0.002–0.055 | |

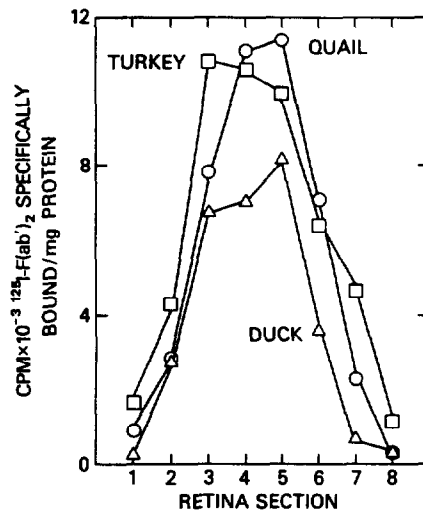


Figure 8
TOP antigen gradients in retina from: (○) Japanese quail (15-day embryo); (Δ) White Pekin duck (16-day embryo); (□) turkey (17-day embryo); the eggs hatch 17, 28, and 28 days after fertilization, respectively. [125 I]F(ab') $_2$ concentrations and μ Ci/pmole were 0.26 nM (0.90 μ Ci/pmole), 0.074 nM (2.92 μ Ci/pmole), and 0.078 nM (2.38 μ Ci/pmole), respectively.

Table 2
Effect of Trypsin, Heat, or Lectins on TOP Antigenicity

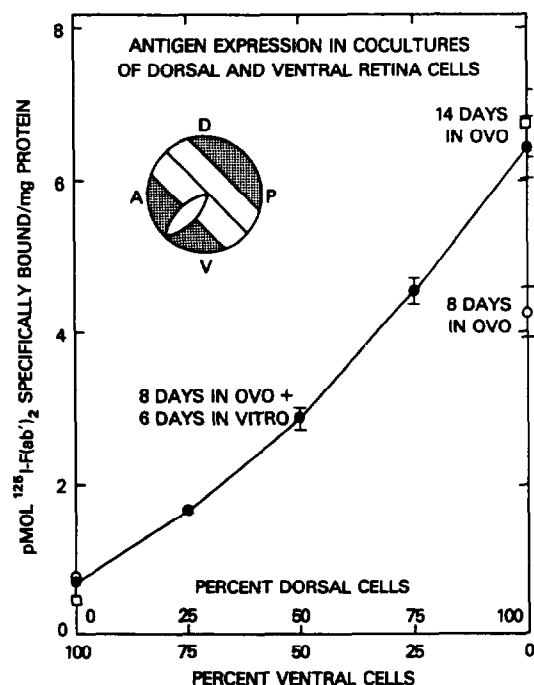
| Exp. no. | Treatment of retina cells | | cpm [125 I]F(ab') ₂ bound specifically to retina cells | % |
|----------|-----------------------------------------------|---------------------|-------------------------------------------------------------------------|-----|
| | 0–30 min | 30–40 min | | |
| 1 | control | + trypsin inhibitor | 1700 | 100 |
| | trypsin | + trypsin inhibitor | 93 | 6 |
| | trypsin + trypsin inhibitor | — | 1803 | 106 |
| 2 | 4°C, 30 min | | 1607 | 100 |
| | 100°C, 30 min | | 132 | 8 |
| 3 | control | | 467 | 100 |
| | 50 μ g wheat germ agglutinin | | 271 | 58 |
| | 50 μ g concanavalin A | | 951 | 204 |
| | 50 μ g <i>Ulex europaeus</i> agglutinin I | | 607 | 130 |
| | | | | |

Properties of TOP antigen molecules in 14-day chick embryo dorsal retina. In experiment 1, retina cells were incubated for 30 min at 37°C, in either phosphate-buffered saline (PBS), PBS with 11 μ M trypsin (crystallized three times; Worthington), or PBS with 11 μ M trypsin inactivated with 12 μ M soybean trypsin inhibitor (Worthington), then for 10 min with soybean trypsin inhibitor. TOP ascites fluid was diluted 1000-fold; 1.86 nM [125 I]F(ab')₂ (9.05×10^{-2} μ Ci/pmole) was used. In experiment 2, TOP and P3-X63/Ag-8 antibodies were diluted 100-fold; 1.82 nM [125 I]F(ab')₂ (9.69×10^{-2} μ Ci/pmole) was used. In experiment 3, retina cells were incubated with lectins (50 μ g per 6×10^6 cells) for 15 min at 4°C, washed to remove unbound lectin, and assayed for TOP. TOP and P3-X63/Ag-8 antibodies were diluted 500-fold.

antibody binding to 58% of the control value, whereas *Ulex europaeus* agglutinin I (specific for L-fucosyl residues) had no effect, and concanavalin A (specific for α -D-mannosyl and α -D-glucosyl residues) increased antibody binding twofold. Increased anti-TOP antibody binding was also observed after treatment with succinylated concanavalin A.

ANTIGEN CONTENT OF CULTURED RETINA CELLS

• Cells dissociated from 8-day chick embryo retina with 0.05% trypsin (crystallized three times), 0.003% collagenase, 2% chick serum, and 0.001% DNase contain little or no TOP antigen; however, cells cultured for 24 hours in vitro bound 50% as much antibody to TOP as did cells from intact retina in ovo. With cells dissociated with trypsin from dorsal or ventral 8-day embryo retina and cultured for 6 days, approximately the same amount of antigen per mg of protein was

**Figure 9**

TOP antigen in monolayer cultures containing varying proportions of cells dissociated with 0.05% trypsin and 0.005% DNase from dorsal or ventral 8-day chick embryo retina and cultured for 6 days. Each 100-mm culture plate contained 8×10^7 retina cells and 15 ml of medium (90% Eagle's minimal essential medium, 10% fetal bovine serum).

detected as with dorsal and ventral 14-day chick embryo retina in ovo (Fig. 9). With trypsinized cells from dorsal and ventral retina that were mixed in different proportions and cocultured for 6 days, antigen levels detected were nearly additive (Fig. 9; linear correlation coefficient, r^2 , 0.98). No evidence of induction or suppression of antigen was obtained under the conditions tested. Dorsal, middle, and ventral 8-day chick embryo retina cells were dissociated with trypsin collagenase, chick serum, and DNase, then cultured for 4 days, dissociated a second time by the same method, and cultured for 6 additional days. The antigen contents detected were similar to those found with dorsal, middle, and ventral retina cells that were dissociated only once and then were cultured for 10 days (Fig. 10). Thus, a gradient of antigen can be reexpressed and maintained for at least 10 days in culture.

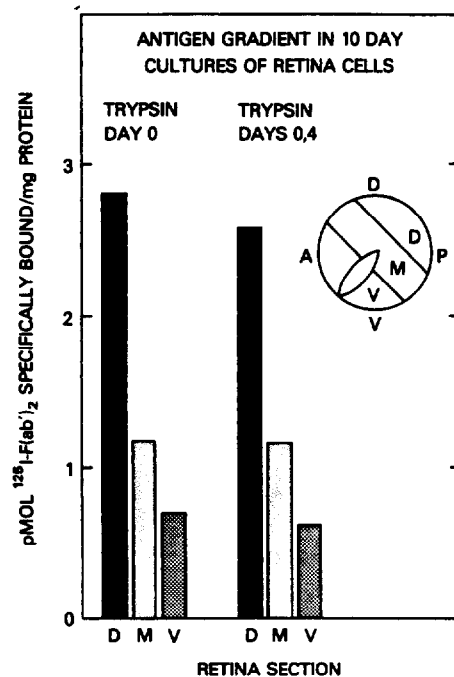


Figure 10

TOP antigen in monolayer cultures containing cells dissociated with 0.05% trypsin, 0.003% collagenase, 2% chick serum, and 0.001% DNase from dorsal, middle, or ventral 8-day chick embryo retina, and cultured for 10 days as in Fig. 9; where indicated, cells were dissociated again as above on the 4th day of culture, recovered, and cultured in fresh 100-mm plates for 6 additional days.

MONOCLONAL ANTIBODIES TO RETINA HYBRID CELL LINES

• As an alternative strategy to identify surface membrane molecules localized to restricted topographic regions of the neural retina, monoclonal antibodies were generated against clonal hybrid cell lines originated by fusing retina cells from rat, mouse, or Chinese hamster embryos with clonal mouse neuroblastoma N18TG2 or human fibroblast VA-2-M7 cells. The retina hybrid cell lines used for immunization of mice were: NCE-9AK (Chinese hamster embryo retina \times N18TG-2), N18RE-103 (Fisher rat embryo retina \times N18TG-2), N18ME-1 (C57/B1 mouse embryo retina \times N18TG-2), and CHEM7A2al (Chinese hamster embryo retina \times VA-2-M7). NCE-9AK, N18RE-103, and

N18ME-1 hybrid cells generate action potentials when stimulated electrically, possess tetrodotoxin-sensitive Na⁺ channels, extend long neurites, and resemble neurons in morphology. N18RE-103 cells synthesize dopamine, and N18ME-1 cells synthesize norepinephrine (E. Heldman et al., unpubl.). In contrast, neither catecholamine synthesis nor tyrosine hydroxylase activity was detected in parental N18TG-2 neuroblastoma cells. NCE-9AK cells possess large, dense-core vesicles and are more adhesive to 10-day chick embryo retina cells than to cerebral cortex cells or N18TG-2 cells (M. Delong and M. Nirenberg, unpubl.).

Spleen cells from immunized mice were fused with P3-X63/Ag-8 mouse myeloma cells, and 1136 hybridoma cell lines were obtained; 133 hybridoma cell lines were obtained that synthesize antibodies that bind to the retina hybrid cells used for immunization (11.7%). Twenty-eight antibodies bound to retina hybrid cells but not to N18TG-2 or VA-2-M7 parental cells or other nonneural cell lines tested from the same species as the retina parental cells.

CONCLUSIONS

- A monoclonal antibody was obtained that defines a cell-surface antigen distributed in a 35-fold concentration gradient in avian retina. The concentration of antigen is highest at the dorsoposterior margin of the retina and lowest at the ventroanterior margin. The antigen was found on most or all cell types in chick retina, and the amount of antigen detected is related to cell position in the retina. The concentration of antigen found is a function of the square of circumferential distance from the ventroanterior pole of the gradient toward the dorsoposterior pole. The dorsoposterior portion of the retina differs from other portions of the retina in neuronal lineage and embryologic development, migration of cells and axons across the midline of the embryo, synapse specificity, adhesive specificity, and function. The function of TOP molecules has not been determined; however, our working hypothesis is that TOP molecules play a role in the encoding or decoding of positional information in the retina. The mechanism of generating and maintaining the highly ordered topographic gradient also remains to be determined.

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